Resonance Raman evidence for an exchangeable protein hydrogen associated with the heme *a* group of cytochrome oxidase

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When cytochrome-*c* oxidase is soaked in D_2O, downshifts of the cytochrome *a* formyl C=O stretching mode are seen in the resonance Raman (RR) spectra (413.1 nm excitation) of both the resting and reduced forms. Other changes observed in the reduced protein RR spectra are consistent with involvement of the cytochrome *a* formyl group in the deuterium effect. The D_2O-induced RR changes are fully developed during 3–5 days incubation, but are incomplete after 1 h. Extraction of the heme *a* chromophore in deuterated solvents eliminates these changes, implying that the exchangeable proton is on a protein group in the cytochrome *a* pocket which H-bonds to the heme formyl. The rate of the D_2O exchange process is unaffected by enzyme turnover, thus reducing the likelihood that the cytochrome *a* formyl H-bond is directly involved in the redox-linked mechanism of proton pumping.

Cytochrome oxidase *Formyl group* *Deuterium exchange* *Resonance Raman spectroscopy*

1. INTRODUCTION

Cytochrome-*c* oxidase is the site of O_2 reduction in the mitochondrial respiratory chain. Four electrons are delivered to O_2 via 4 metal centers: cytochrome *a* and a_3, and two copper ions, Cu_A and Cu_B. In addition, the enzyme is responsible for translocating protons across the inner mitochondrial membrane [1–3]. Raman spectra obtained with visible laser excitation, near resonance with the strong porphyrin π–π^* electronic transitions, contain numerous bands associated with porphyrin vibrational modes. Both heme cofactors contribute to these spectra, but considerable progress has been made towards assigning the individual heme bands through systematic studies reported from several laboratories [4–8].

Of particular interest is the C=O stretching vibration of the formyl peripheral substituent, which is conjugated with the porphyrin π electronic system. For high-spin cytochrome a_3 constituents this mode is found at 1676 and 1668 cm^{-1} in the resting and reduced forms of the enzyme, consistent with the frequencies observed for high-spin heme *a* model compounds [4]. For the low-spin cytochrome *a* constituents, however, the C=O frequencies are lower, by 20–35 cm^{-1}, than expected on the basis of model compounds, and have been tentatively assigned at 1650 and 1611 cm^{-1} for the resting and reduced forms; the frequency decreases, relative to model compounds, have been suggested to reflect strong H-bonding to the formyl O atom from a protein donor [9,10]. Babcock and Callahan [10] have suggested that the protein donor group may be involved in proton pumping in cytochrome oxidase, since the H-bonding appears substantially stronger in the reduced protein.

In this paper data are presented which confirm the existence of the H-bond between a protein

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donor and the formyl of cytochrome *a* via a D_2O-induced downshift of the cytochrome *a* formyl C=O stretch. H/D exchange at the protein donor group is established by the loss of the effect when the heme chromophore is isolated from the protein. This group may not participate directly in proton pumping, however, since no acceleration of exchange is observed when the enzyme turns over.

2. EXPERIMENTAL

Cytochrome-*c* oxidase was isolated from bovine hearts as in [11,12] and dissolved in 50 mM Hepes (Sigma) buffer, pH 7.4, 0.5% lauryl maltoside (Cal Biochemical) to a concentration of 150–200 μM in oxidase. All samples contained 0.3 M sodium sulfate as an internal frequency/intensity standard for Raman spectroscopy. D_2O exchange was accomplished by concentrating 2 ml of sample (centrifugation at 4°C in an Amicon Centricon™ concentrator with membrane cutoff = 10 kDa) to 40 μl with deuterated buffer, and repeating the process three times. The samples were incubated in the resting form at 4°C until acquisition of Raman spectra. Control samples (aqueous buffer exchanged for aqueous buffer) were treated in a similar fashion. Buffer pH/p*D* values were measured with a Corning 150 pH meter. The p*D* values were corrected for the deuterium isotope effect [13]. Reduced enzyme was prepared by addition of a few grains of sodium dithionite (Aldrich) to the resting form and then monitored spectrophotometrically. Enzyme turnover was carried out by mixing completely reduced samples vigorously with air to form the 'reoxygenated form' and rereducing with additional dithionite [14–16].

The heme *a* chromophores were isolated from the bovine enzyme, and the Fe^{II} heme *a*-bispyridine complex was prepared as in [17]. The concentration of the complex was determined spectrophotometrically [18].

The Raman spectra were obtained using 413.1 nm excitation as described [19]. The high-frequency spectra were collected at 1 cm^{-1}/s increments, with 5 cm^{-1} spectral resolution, and are each the sum of 5 consecutive scans. The low-frequency spectra were collected at 0.25 cm^{-1}/s increments, with 3 cm^{-1} spectral resolution, and are the sum of 12 consecutive scans.

3. RESULTS AND DISCUSSION

Fig.1 shows 413.1 nm RR spectra in the C=O stretching region for the resting and reduced enzyme in H_2O and after soaking for 3–5 days in D_2O. The cytochrome a_3 C=O modes, at 1676 and 1668 cm^{-1} [11], are unaffected by D_2O, but definite downshifts are seen for the bands assigned to the cytochrome *a* C=O modes. The resting enzyme in H_2O shows bands at 1640 and 1650 cm^{-1} which have been assigned to cytochrome *a* skeletal mode ν_{10} and the C=O stretch, respectively [4,10]. In D_2O a band is seen at 1646 cm^{-1}, with a shoulder at 1640 cm^{-1}, indicating that the 1650 cm^{-1} C=O stretch has shifted down by 4 cm^{-1}. The reduced protein shows a broad band at 1620 cm^{-1} in H_2O, due to overlapping contributions from the cytochrome *a* ν_{10} and C=O stretch which are seen as separate bands, at 1622 and 1611 cm^{-1} with 441.6 nm excitation [5]. In D_2O this band is clearly resolved into two components,

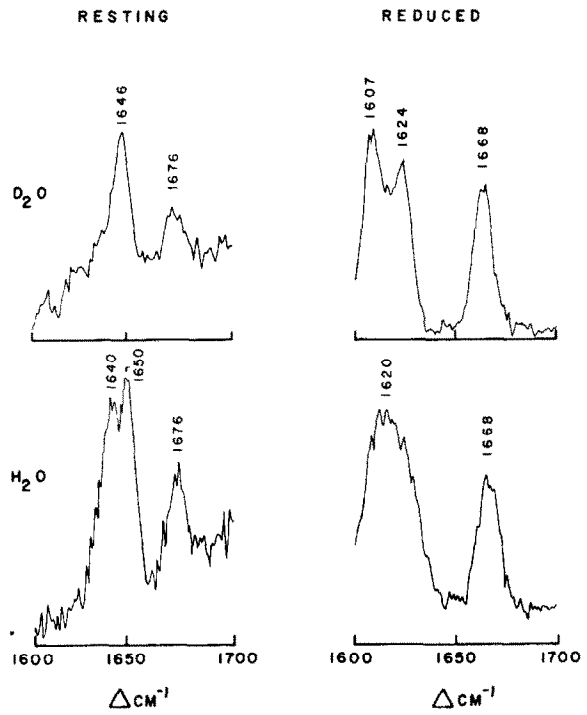

Fig.1. High-frequency RR spectra of resting (left panel) and reduced (right panel) cytochrome-*c* oxidase in H_2O (bottom) and D_2O (top) in the region of the cytochrome *a* and a_3 formyl C=O stretching bands.

at 1607 and 1622 cm^{-1}. Thus there is a 4 cm^{-1} downshift in the C=O stretch, 1611⟶ 1607 cm^{-1}, in the reduced protein as well as in the resting form.

Two explanations for these shifts can be considered: (i) H/D exchange of the cytochrome *a* formyl proton (catalyzed presumably by a protein residue since this is otherwise a very slow process) which alters the coupling between C-H bending and C=O stretching modes. Approx. 5 cm^{-1} downshifts in $\nu_{C=O}$ have been reported for model heme *a* complexes and benzaldehyde upon deuteration of the formyl proton [4]. (ii) H/D exchange of a proton involved in H-bonding to the formyl O atom, producing a $\nu_{C=O}$ shift due to the stronger D····O bond (lower zero point energy). The extent of this shift, which increases with increasing H····O bond strength, is difficult to gauge. We note that 2–4 cm^{-1} D_2O shifts have been reported for the O-O frequency of Co substituted hemoglobin [20] and the C-O frequency of the CO adduct of horseradish peroxidase [21] and cytochrome-c peroxidase [22]. To distinguish between these possibilities the hemes were extracted from enzyme equilibrated with D_2O and H_2O, and the RR spectra of their bispyridine complexes were recorded. If the cytochrome *a* formyl proton had exchanged, there should have been an ~5 cm^{-1} C=O downshift for 50% of the extracted heme. Fig.2 shows, however, that there is no difference in the C=O frequency or the width of the band for heme extracted from enzyme in H_2O or D_2O. This experiment strongly supports the inference [9] that the cytochrome *a* formyl group is strongly H-bonded by a protein residue.

Other effects are seen in the reduced cytochrome-*c* oxidase RR spectra which are consistent with formyl perturbation via H-bonding. In the region below 750 cm^{-1} (fig.3) two D_2O-induced alterations are seen: (i) The 350 cm^{-1} band gains intensity relative to its 343 cm^{-1} neighbor. The two bands are believed to be associated with a Fermi resonance between the ν_8 porphyrin deformation mode and the overtone of another porphyrin mode, ν_{35} [23]. Both of these involve contributions from peripheral substituents, possibly including the formyl group, and a slight frequency

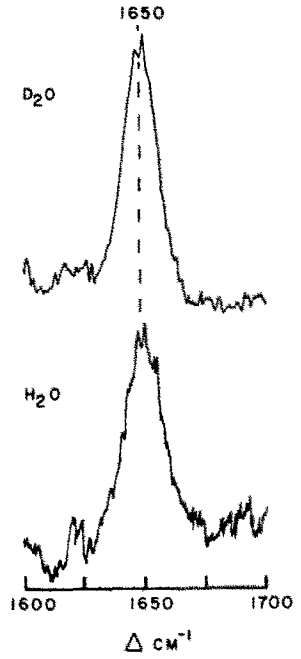

Fig.2. RR spectra, in the formyl C=O stretching region, of heme *a* bispyridine isolated from cytochrome-*c* oxidase which had been incubated in H_2O (bottom) and D_2O (top).

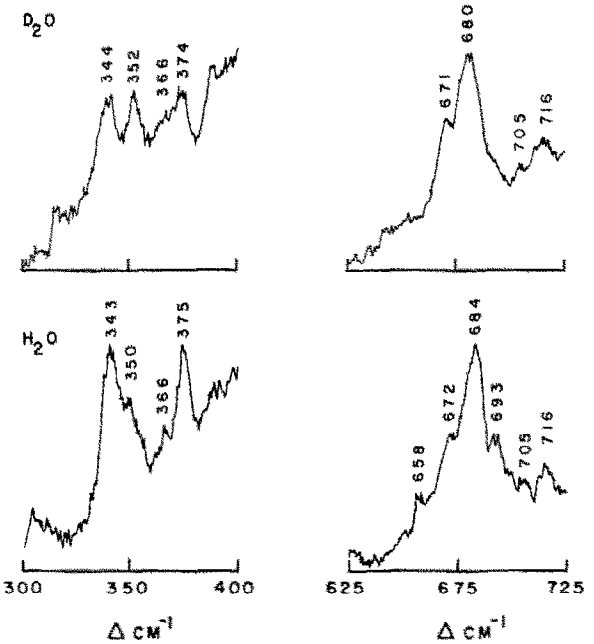

Fig.3. Low-frequency RR spectra of reduced cytochrome-*c* oxidase in H_2O (bottom) and D_2O (top).

change in either would appear as a change in the Fermi doublet intensity ratio. (ii) A previously unreported band at 693 cm^{-1} shifts under its neighboring 684 cm^{-1} band, leading to an apparent 4 cm^{-1} downshift of the latter in D_2O. We tentatively assign this band to the porphyrin-C-O bend of the formyl group; this mode is observed at 649 cm^{-1} in benzaldehyde [24]. In the mid-frequency region (fig.4), the 1293 cm^{-1} band appears to split into two components in D_2O. This band was previously assigned to ν_{42} [4], but the present results suggest some contribution from the ring-formyl stretch. A slight shift of the ring-formyl stretch in cytochrome *a* but not a_3 would account for the observed split.

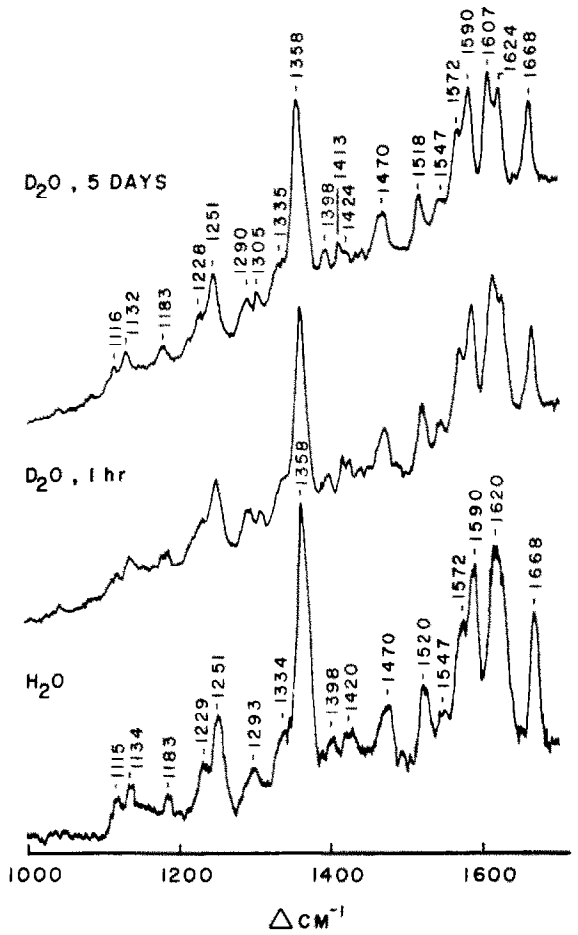

Fig.4. RR spectra of reduced cytochrome-*c* oxidase in H_2O and after incubation in D_2O for various times. From bottom to top: in H_2O, in D_2O after 1 h incubation and in D_2O after 5 days incubation.

Fig.4 also shows that the formyl C=O frequency shift is detectable after 1 h in D_2O but is incomplete. 3–5 days of soaking were required to develop the shift completely. Capaldi [25] monitored peptide H/D exchange in cytochrome-*c* oxidase with IR spectroscopy and found that 72 h are required for exchange to level off; the slower exchanging protons were suggested to be buried in hydrophobic regions or stabilized by H-bonds. The evidence is that cytochrome *a* is not buried deeply in the protein, but lies close to the cytochrome *c* binding surface [1]. Ca^{2+} effects on cytochrome *a* suggest that it is in a solvent exposed environment [26]. Thus the slow H/D exchange rates seen in the RR experiments are again consistent with strong H-bonding of the affected proton. Babcock and Callahan [10] have estimated the strength of this bond to be 3.0 and 5.3 kcal/mol for the resting and reduced enzyme, respectively.

To test the idea that this H-bond might be an integral part of the proton pumping mechanism, we carried out an experiment to determine whether exchange is accelerated by enzyme turnover. If, as

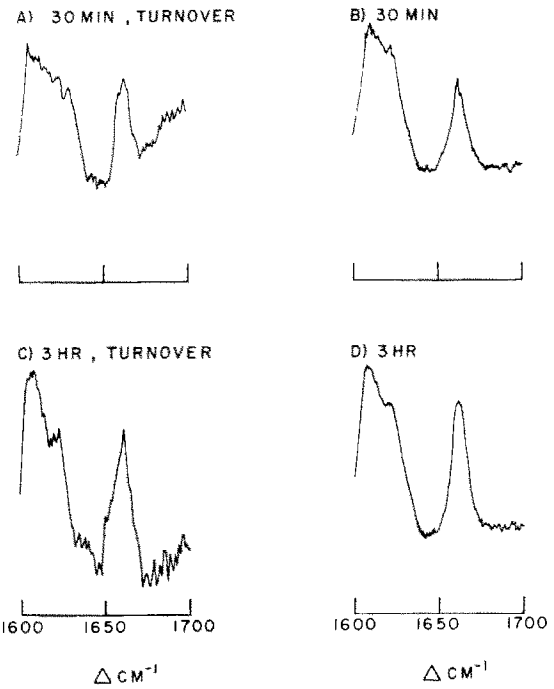

Fig.5. High-frequency RR spectra of reduced cytochrome-*c* oxidase in D_2O with (A,C) and without (B,D) enzymatic turnover. Spectra were taken 30 min after turnover (A,B) and again 3 h after turnover (C,D).

Babcock and Callahan have suggested [9,10], the protein H-bonding group acts as donor and acceptor to the protons involved in the pump, which is driven by the H-bond energy difference between resting and reduced cytochrome *a*, then H/D exchange should occur every time the enzyme turns over. In the experiment enzyme in the presence of excess dithionite was exposed to sufficient air to turn it over many times. The result, however, was negative, as shown in fig.5. There was no significant increase in the rate of H/D exchange. While the H-bond to the formyl group may provide an interaction that assists proton pumping, a direct role appears to be precluded.

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